Available online at www.sciencedirect.com

ScienceDirect

Procedia Food Science 3 (2015) 453 – 464

Procedia Food Science

The First International Symposium on Food and Agro-biodiversity (ISFA2014)

The use of Red Oncom Powder as Potential Production Media for Fibrinogenolytic Protease Derived from *Bacillus licheniformis* RO3

Diana Nur Afifah[a,b], Muhammad Sulchan[a], Dahrul Syah[b], Yanti[c], Maggy T. Suhartono[b]

[a]*Department of Nutrition Science, Faculty of Medicine, Diponegoro University, Semarang, Indonesia.*
[b]*Department of Food Science and Technology, Faculty of Agricultural Technology, Bogor Agricultural University, Bogor, Indonesia.*
[c]*Faculty of Biotechnology, Atma Jaya Catholic University, Jakarta, Indonesia.*

Abstract

The high cost of enzyme production is one of the barriers to successful application of enzyme in the industry. Selection of media is a critical factor for the enzyme production. Main factors for optimization of enzyme production include nutritional components and environmental conditions for growth and production of fibrinogenolytic protease. In this study, *Bacillus licheniformis* RO3 isolated from *red oncom*, an Indonesian fermented food was tested for its fibrinogenolytic protease production by using several media. Three types of media were analyzed, i.e. Luria-Bertani broth (LB), ½ LB + 1% skim milk (LBS), and ½ LB + 1% *red oncom* powder (LBO). Protease activity was tested by using spectrophotometric method with casein as a substrate and fibrinogenolytic activity was confirmed based on zymography assay using fibrinogen substrate. In LB media, *B. licheniformis* RO3 was able to produce protease with activity of 0.024 U/ml or 0.157 U/mg at 36 h fermentation. In LBS media, the highest protease activity was 0.022 U/ml or 0.152 U/mg at 48 h fermentation. The best result was shown by *B. licheniformis* RO3 grown in LBO media with the highest protease activity of 0.051 U/ml or 0.283 U/mg at 48 h. Zymographic profiles showed that crude enzyme from *B. licheniformis* RO3 consisted of six fibrinogenolytic bands with molecular weight of 20, 27, 32, 40, 70, and >140 kDa. These results indicate that *red oncom* powder can be used as a potential media for fibrinogenolytic protease production.

Peer-review under responsibility of the organizing committee of Indonesian Food Technologist Community

Keywords: *red oncom* powder, fibrinogenolytic protease, production media, *B. licheniformis*

* Corresponding author.
E – mail address : mthenawidjaja@yahoo.com

Peer-review under responsibility of the organizing committee of Indonesian Food Technologist Community
doi:10.1016/j.profoo.2015.01.050

Introduction

The high cost of enzyme production is one of the barriers of the success of protease application in the industry. The selection of production media is a critical factor for fibrinogenolytic protease fermentation. Fibrinogenolytic protease-producing microbes have various physiological characteristics, then, the optimization of nutritional components and environmental conditions is needed for the growth and production of enzymes [1,2]. Fibrinolytic and fibrinogenolytic enzymes are protease enzyme group which are able to degrade fibrin or fibrinogen. In the body, the fibrinolytic enzyme or plasmin is produced by endothelial cells in the pancreatic duct. Along with the increasing age and the imbalance pattern of food consumption, the production of natural plasmin by body will be reduced that may cause to the disruption of fibrinolytic system. If it goes on a regular basis, it will lead to the thrombosis related-degenerative diseases, such as stroke, atherosclerosis, hypertension, and diabetes.

The *Bacillus* genus from fermented foods is one of microbes that is found to be able to produce a strong fibrinolytic enzyme, such as *Bacillus* natto from *natto*, a traditional Japanese soy fermented food that produce nattokinase (NK). The supply of *natto* or its enzyme orally is not only able to degrade the thrombus directly, but also able to increase the release of endogenous plasminogen activator in experimental animals and human subjects [3]. Other *Bacillus* genus of various fermented foods have also been found to be able for producing strong fibrinolytic enzymes, including *B. amyloliquefaciens* DC-4 from *douchi*, a fermented soy food from China [4], *Bacillus* sp. CK from *chungkookjang*, a fermented soy sauce from Korea [5], *Bacillus* sp. DJ-2 strains and DJ-4 from doenjang, a Korean fermented soybean paste [6,7], and *Bacillus* sp. KA38 from *jeotgal*, a fermented salted fish from Korea [8].

Oncom is one of traditional Indonesian fermented food in West Java. This food is made from a fermentation process generated by several fungi. There are two types of *oncom*, i.e. *red oncom* and *black oncom*. The *red oncom* is degraded by the fungus of *Neurospora sitophila* or *N. intermedia* while the *black oncom* is degraded by the fungus of *Rhizopus oligosporus* and other types of *Mucor* [9,10]. Generally, *red oncom* is made from tofu waste, i.e. the soy which its protein has been taken in its make, while the *black oncom* is generally made from the peanuts dregs mixed with cassava dregs or cassava powder, i.e. tapioca, in order to make a better texture and more tender. Although both the substrate material is a kind of waste, its nutrient is still high enough to be exploited by human.

Tofu waste still contains high nutrient values, however, most of its organoleptic properties are less preferred. Tofu waste with a fermentation process, i.e. *red oncom*, is preferred as food product than its original waste without fermentation. Tofu waste is a processed product from tofu in which

its protein nature is probably similar to tofu and soy, although it has undergone many changes because of certain treatments during the manufacturing process of tofu, such as heating. The high nutrient content of tofu and its large amounts provide a significant opportunity to be used as a growth media for enzymes-producing microbes for health. Therefore, this study was focused for selecting the optimal production media for production of fibrinogenolytic protease by fibrinogenolytic protease-producing microbe, i.e. *Bacillus licheniformis* RO3 isolated from *red oncom*, an Indonesian fermented food. Instead of commercial media of LB and skim milk, the use of *red oncom* powder as the alternative media for enzyme production was also tested since the microbe tested was originally isolated from fresh *red oncom*. Fibrinogenolytic protease with the highest activity was further characterized for its optimum pH and temperature.

Materials and Methods

Microorganism

Microbe tested was *B. licheniformis* RO3 isolated from traditional fermented food of fresh *red oncom* [11].

Production of fibrinogenolytic protease

A total of 2 ose microbes was grown in 25 mL sterile Luria-Bertani broth media (LB) to obtain the optical density value (OD) of 0.8 at λ_{620nm}. LB media contained isolates that had reached a value of OD = 0.8 and then it was taken 10% to be added in three different media, LB, ½ LB + skim milk 1% (w/v), herein after referred to as LBS, and ½ LB + *red oncom* powder 1% (w/v), herein after referred to as LBO. Microbial incubation was performed for 72 hours at 37°C in a waterbath at 120 rpm. OD_{620} value was measured every 12 hours to determine the growth curve of microbes. The production media that has contained with fibrinolytic protease was taken once every 12 hours and centrifuged at 6000 g for 15 minutes at 4°C. The supernatant was taken to be calculated for its fibrinogenolytic protease activity.

Analysis of protease activity

Protease activity was quantitatively measured by a modification of Bergmeyer and Grassl method [12] using Hammarsten casein substrate (1% w/v). Three steps of analysis treatment were conducted, including the blank, standard, and samples. Enzyme solution that was heated to a certain temperature and incubation time (which produces maximum activities) was added to microtube containing 250 µL of 50 mM phosphate buffer pH 8. For treatment of blank and standard, the enzyme was replaced with distilled water and 5 mM tyrosine.

The solution was incubated at 37°C for 10 minutes. The hydrolysis reaction was stopped by the addition of 500 µL of trichloroacetic acid (TCA) 0.1 M. Blank and standard were added with 50 µL of enzyme solution, while sample was added with 50 µL of distilled water, and then the solution was reincubated at 37°C for 10 minutes, followed by centrifugation at 4000 g and temperature of 4°C for 10 minutes. Briefly, a 375 µL of supernatant was added to a microtube containing 1.25 mL Na_2CO_3 0.4 M and the Folin Ciocalteau reagent, and then incubated again at 37°C for 20 minutes. The absorbance of the solution was measured at 578 nm.

Analysis of protein concentration

The protein concentration was determined by Bradford method [13] using bovine serum albumin (BSA) as protein standard. A total of 100 µL enzyme was added to the tube containing 1 mL distilled water and 1 mL Bradford reagent. For blank treatment, the enzyme solution was replaced with distilled water. Then, the solution was mixed using vortex and allowed to stand for 20 minutes at room temperature. Absorbance of the solution was measured at a wavelength of 595 nm. For standard, enzyme solution was replaced with BSA Fraktion V with concentration ranges of 0-250 µg/mL. The protein concentration of enzyme solution was determined by linear equation of relationship between standard concentration of protein and absorbance.

Activities of fibrinogenolytic with zymography

Zymography was performed to detect fibrinogenolytic activity directly using fibrinogen substrate. Several steps in zymography included the preparation of separating and stacking gels, sample and loading preparation, running condition, gel staining, gel destaining, and visualization. Separating gel was made from a 12% acrylamide concentration. Enzyme was dissolved in sample buffer containing 2-mercaptoethanol with no heating treatment. Each sample was injected into a gel well with the volume range of 10-20 µL. Electophoresis was run at a voltage of 70 V, 50 A for 2-3 hours in an electophoresis buffer. Furthermore, the gel was first denatured in a solution of Triton X-100 2.5% v/v and shaked for one hour. Gel was digested in 50 mM universal buffer pH 7 and temperature of 37°C for 12 h. Gel was stained with staining solution (*Coomassie Brilliant Blue* R-250) for 15 minutes. Gel was destained with destaining solution repeatedly until a white fibrinogenolytic enzyme band was resulted with a blue gel background.

Characterization of enzyme

Characterization of fibrinogenolytic protease including optimal pH and temperature was tested quantitatively by spectrophotometric method [12]. Optimization of enzyme pH was carried out at 37°C by means of the analysis of enzyme activities using universal buffers with pH range of 2-12. Determination of the optimum temperature was done by measuring the enzyme activity at various temperatures (30-80°C) in universal buffer of pH 7.0.

Results and Discussion

Media selection and growth rate were conducted to determine the optimal condition for *B. licheniformis* RO3 to produce high fibrinolytic protease. The use of *red oncom* powder as an alternative production media for microbe growth was due to the original source of the microbe that isolated from the fresh *red oncom*. It may be developed for enzyme production media with low cost, safe, and high enzyme activity. Also, it may be potentially used for the development of the functional foods derived from the *red oncom.*

Fig. 1 showed that the *B. licheniformis* RO3 in LB media grew exponentially until 36 hour incubation and continued to stationary phase until 60 hour of growth rate. At 72 hour incubation, the microbial growth has not decreased yet. This result was not linear with the microbial growth in LBS media. *B. licheniformis* RO3 grew exponentially up to 12 hour incubation and entered the stationary phase after 24 hour incubation. In contrast, in the LBO media, B. *licheniformis* RO3 grew rapidly until the 48 hour incubation and constantly continued in the stationary phase until the 72 hour incubation.

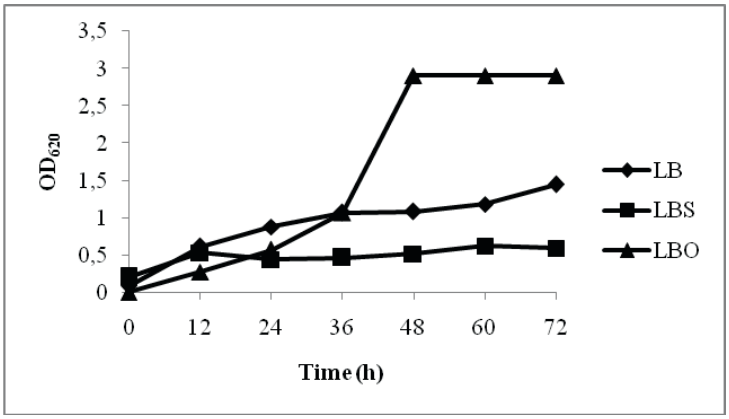

Fig. 1. The growth curve of *B. licheniformis* RO3 on different media: Luria-Bertani broth (LB), ½ LB + 1% skim milk (LBS), and ½ LB + 1% red oncom powder (LBO)

Fig. 2 showed protease activities (**A**) and specific activities (**B**) of *B. licheniformis* RO3 in various production media. In the In LB media, *B. licheniformis* RO3 was able to produce protease enzyme with the highest activity of 0.024 U/ml or 0.157 U/mg after 36 hours incubation. Meanwhile, in LBS media, *B. licheniformis* RO3 produced protease enzyme with the highest activity of 0.022 U/ml or 0.152 U/mg at 48 hours incubation. These results indicate that the reduction of composition of LB media and the addition of skim milk to the production media may be not significant to increase the protease activities, but only extend the production rate. It seems that skim milk is not suitable for induction of *B. licheniformis* RO3 in producing protease enzyme.

B. licheniformis RO3 LBO grown in LBO media was potentially to produce enzyme with highest protease activity (0.051 U/ml or 0.283 U/mg) at 48 hours incubation. It is noted that the enzyme activity produced by microbes in the LBO media significantly increased twice compared to those of LB and LBS media. These results indicate that the microbes grown in original media may be more adaptable to produce extracellular enzymes with high activity and optimum characters.

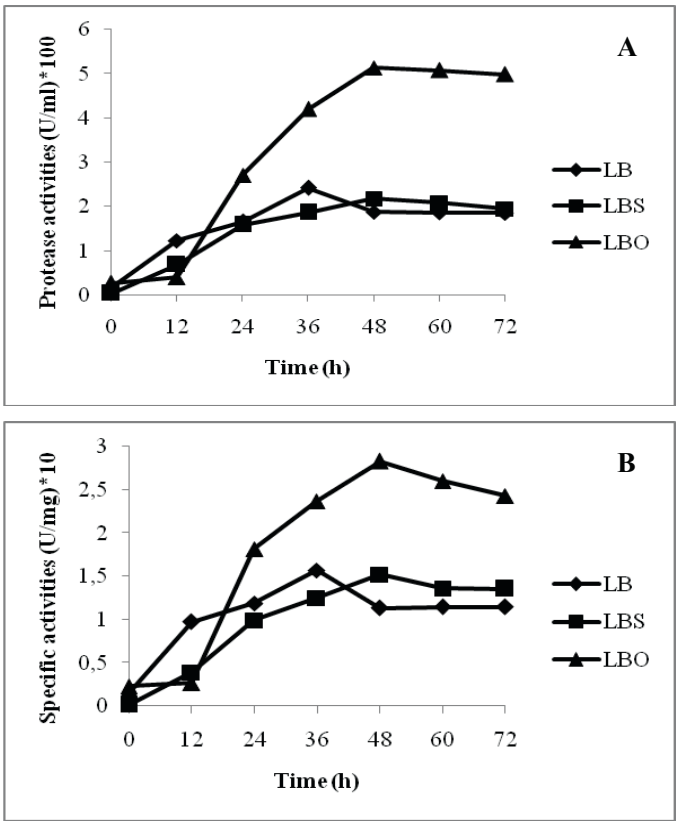

Fig. 2. Protease activities (**A**) and specific activities (**B**) of B. *licheniformis* RO3 on different media: Luria-Bertani broth (LB), ½ LB + 1% skim milk (LBS), and ½ LB + 1% red oncom powder (LBO)

The relationship between the growth patterns of microbial cells and protease activities showed that the optimum protease activities of *B. licheniformis* RO3 were achieved when entering the stationary phase during growth production in LB, LBS, or LBO media. In LBO media, B. *licheniformis* RO3 grew rapidly after 48 hours incubation, then the growth was tend to be constant until 72 hours incubation. Compared to other study, *B. licheniformis* Lbbl-11 isolated from *iru* produced maximum extracellular protease at 48 hours incubation or at the stationary phase when grown in nutrient broth media [14].

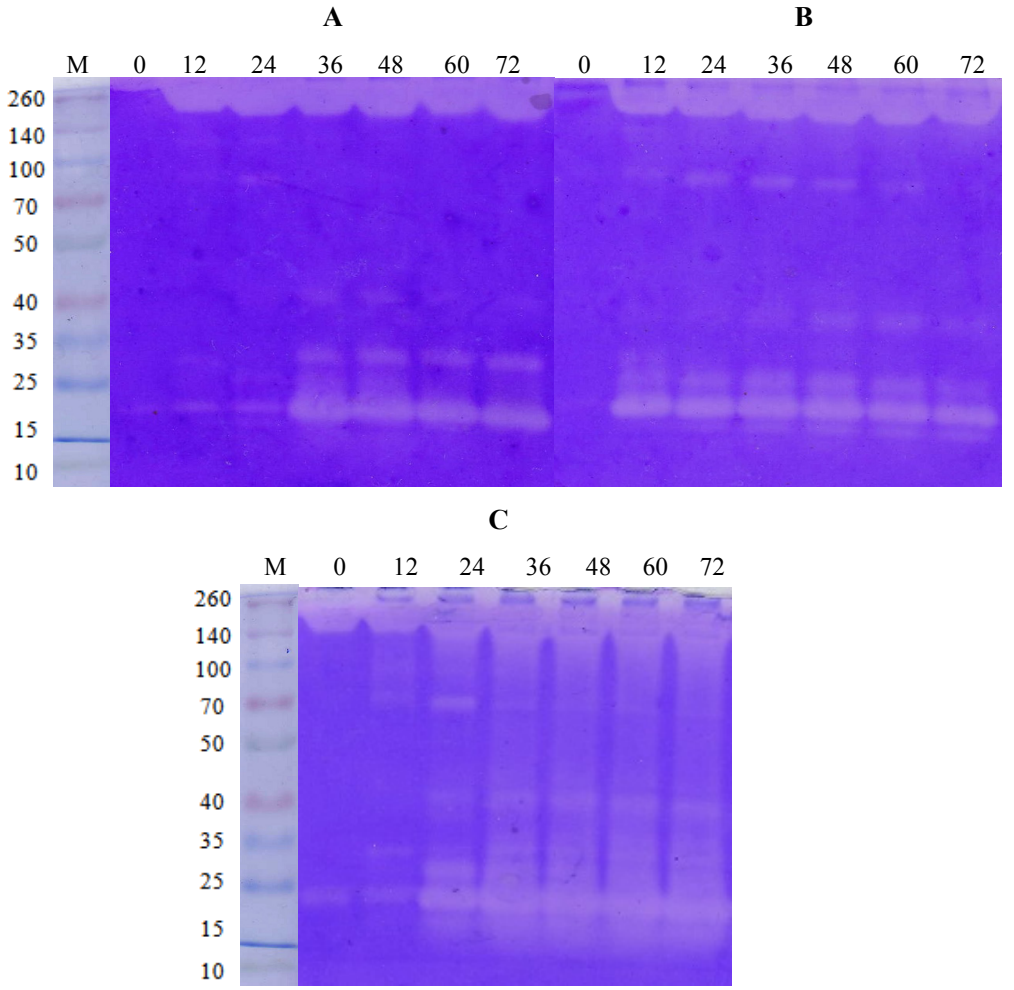

Fig. 3. Activity of fibrinogenolytic with 0.1% fibrinogen substrate in LB media (**A**), LBS (**B**), and LBO (**C**) starting from 0-72 hours incubation. Enzyme digestion was run for 12 h incubation at 37^{o}C.

Activities of fibrinogenolytic from *B. licheniformis* RO3 were determined qualitatively by conducting zymographic method using fibrinogen substrate. Fig. 3 showed that in LB media,

fibrinogenolytic fractions with molecular weight (MW) of <35 kDa from *B. licheniformis* RO3 crude were clearly visible starting from 36 hours incubation. While in LBS media, at 12 hours incubation, fibrinogenolytic fractions with MW of <35 kDa from *B. licheniformis* RO3 crude had been visually seen. Fibrinogenolytic fractions of *B. licheniformis* RO3 grown in LBO media was clearly visible after 24 hours incubation and increasing steadily up to the 72 hours incubation. Zymographic results demonstrated that crude enzyme from *B. licheniformis* RO3 had six fibrinogenolytic bands with molecular weight of 20, 27, 32, 40, 70, and > 140 kDa.

Study on fibrinolytic enzyme isolated from *B. licheniformis* KJ-31 from Hwang *et al.* [15] represented a high enzyme activity (12.8 U/mg) when it was grown in LB media for 72 hours incubation. Fibrinolytic enzyme from *B. polymaxa* NRC-A also exerted a high activities after being grown for 3 days [16]. Interestingly, study of Kim *et al.* [17] demonstrated the use of 4 different production media, including triptic soy broth (TSB), Luria-Bertani broth (LB), nutrient broth (NB), and brain heart infusion (BHI) for growing *B. amyloliquefaciens* CH51. Among all media, the microbe growth in TSB media showed the highest fibrinolytic activity (>2.5 U/ml) after 48 hours incubation.

Some starches and dextrin have been reported as the best carbon sources for *B. amyloliquefaciens* DC-4 in producing the fibrinolytic enzyme [4]. Other study from Agrebi *et al.* [18] reported that *B. subtilis* A26 exerted potential fibrinolytic enzyme in the medium containing 40.0 g/L wheat, 3.53 g/L casein peptone, 4.0 g/L CaCl 2, 3.99 g/L NaCl, 0.01 g/L MgSO 4, and 0.01 g/L KH_2PO_4, pH 7.78. Optimization of media significantly affected the increase of fibrinolytic enzyme production up to 4.2 times (269.36 U/ml) compared with those obtained with the initial media (63.45 U/ml).

B. subtilis Natto B-12 produced the highest nattokinase activities when maltose was used in the production media [19]. Conversely, the enzyme production was very low when using sucrose as carbon source. High concentration of sucrose inhibited microbial action to produce protease, whereas the use of maltose lowered catabolite repression and induced the production of enzymes. Carbamide and ammonium sulfate decreased the production of nattokinase. Carbon source such wheat bran was also chosen to increase the enzyme yield. Bran is composed of starch (12-18%), protein (15-18%), dietary fiber (35-50%), fat (3-5%), and ash (4-6%). Wheat bran can enrich aminophenol, vitamins, minerals, and enzymes [19]. Therefore, the optimal media composition for producing nattokinase from *Bacillus subtilis* Natto B-12 was maltose 2%, 3% wheat bran, 0.5% NaCl, 0.1% KH_2PO_4, 0.4% K_2HPO_4, and $MgSO_4.7H_2O$ 0.05%, pH 7.0 [19].

In this study, the *red oncom* powder as the alternative medium for enzyme production from *B. licheniformis* RO3 contained 23.2% protein, 3.5% fat, 62.3% carbohydrate, and 4.95% ash. Our

results (Fig. 2 demonstrated that among all 3 production media, the LBO media containing red oncom powder significantly increased fibrinogenolytic protease activities due to its high contents of protein and fiber. Fiber component is known as the main contributor to the high carbohydrate content in the *red oncom* powder. Meanwhile, high protein content in the *red oncom* powder can be used as a substrate for microbial *B. licheniformis* RO3 to produce the fibrinolytic protease with high acitivity and optimal characters.

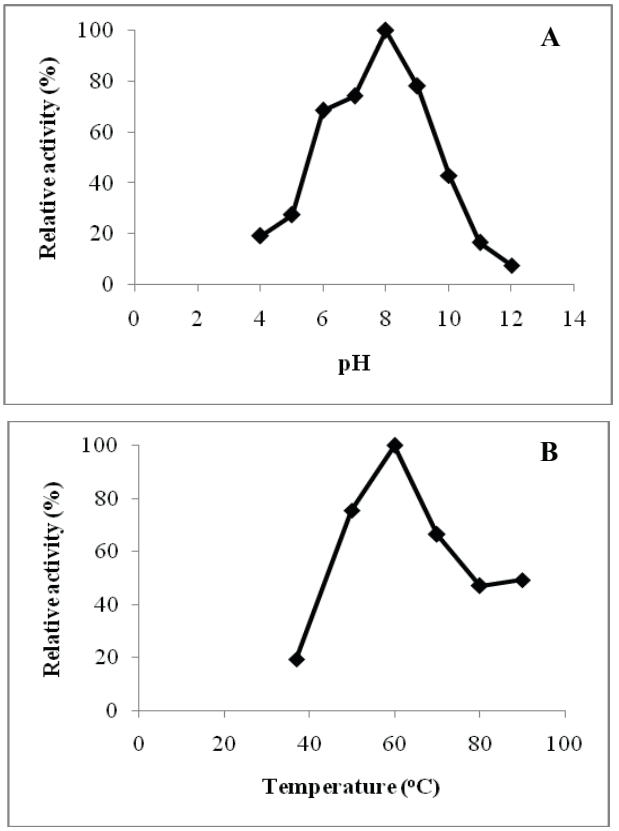

Fig. 4. The effect of pH (**A**) and temperature (**B**) of the fibrinogenolytic protease from *B. licheniformis* RO3 grown in LBO media for 48 h incubation

Fibrinogenolytic protease produced from *B. licheniformis* RO3 in LBO media had an optimum pH of 8 and optimum temperature of 60°C (Fig. 4). Enzyme action is strongly influenced by its optimal pH and temperature. Protease characteristics from *B. licheniformis* have been widely reported. For example, *B. licheniformis* Lbbl-11 isolated from *iru*, a fermented food from Africa, was able to produce extracellular proteases that had optimum temperature of 60°C and optimum pH of 8 [14]. It is also reported that most fibrinolytic enzymes grouped in serine protease were

generally active at neutral and alkaline pHs, with the optimum pH 8 and 10 [7,20]. The optimum temperature of enzymes showed a wide range working temperatures from 30°C to 70°C [5,7], but mostly at 50°C [21,22].

Conclusion

Original source of isolated microbes can be used as the alternative production media to produce specific enzymes. *B. licheniformis* RO3 isolated from traditional fermented food of fresh *red oncom* was successfully grown in production media containing *red oncom* powder (LBO media) with potential fibrinogenolytic protease activities compared to other commercial media. Crude fibrinogenolytic protease enzyme from *B. licheniformis* RO3 had an optimum pH and temperature at 8.0 and 60°C.

Acknowledgment

This work was supported by Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia.

References

[1] Lee J, Park S, Choi WA, Lee KH, Jeong YK, Kong IS, Park S. Production of a fibrinolytic enzyme in bioreactor culture by *Bacillus subtilis* BK-17. *J Microbiol Biotechnol* 1999;9(4):443-449.

[2] Seo JH, Lee SP. Production of fibrinolytic enzyme from soybean grifts fermented by *Bacillus firmus* NA-1. *J Med Food* 2004;7(4):442-449.

[3] Sumi H, Hamada H, Nakanishi K, Hiratani H. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.* 1990;84:139-143.

[4] Peng Y, Zhang YZ. Optimation of fermentation conditions of douchi fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4. *Chin J Appl Environ Biol* 2002;8:285-289.

[5] Kim W, Choi K, Kim Y. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. Strain CK 11-4 screened from *Chungkookjang*. *Appl Environ Microbiol* 1996;62:2482-2488.

[6] Choi NS, Yoo KH, Hahm JH, Yoon KS, Chang KT, Hyun BH, Maeng PJ, Kim SH. Purification and characterization of a new peptidase, bacillopeptidase DJ-2, having fibrinolytic activity: produced by Bacillus sp. DJ-2 from Doen-Jang. *J Microbiol Biotechnol* 2005;15(1): 72–79.

[7] Kim SH, Choi NS. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp. strain DJ-4 screened from Doen-Jang. *Biosci Biotechnol Biochem* 2000;64:1722–1725.

[8] Kim HK, Kim GT, Kim DK, Choi WA, Park SH, Jeong YK, Kong IS. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. KA38 originated from fermented fish. *J Ferment Bioeng* 1997;84(4): 307–312.

[9] Sastraatmadja DD, Tomita F, Kasai T. Production of high-quality oncom, a traditional Indonesian fermented food, by the inoculation with selected mold strains in the form of pure culture and solid inoculum. *J Grad Sch Agr Hokkaido Univ* 2002;70:111-127.

[10] Wood BJB. (ed). Microbiology of Fermented Foods Vol. 2. 2nd ed. Blackie Academic and Professional. 1998;pp 498.

[11] Afifah DN, Sulchan M, Syah D, Yanti, Suhartono MT. Proteolytic and fibrinolytic activities of several microorganisms screened from Red Oncom and Gembus, Indonesian fermented soybean cakes. Abstract presented at 4th Annual International Symposium on Wellness, Healthy Lifestyle and Nutrition. Yogyakarta, Indonesia. 2013.

[12] Bergmeyer HU, Grassl M. Methods of Enzymatic Analysis. Vol ke-2. Weinheim: Verlag Chemie. 1983;pp 1007-1009.

[13] Bradford MM. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein dye-binding. *Anal Biochem* 1976;72:234-254.

[14] Olajuyigbe FM, Ajele JO. Some Properties of Extracellular Protease from *Bacillus licheniformis* Lbbl-11 Isolated from "iru", A Traditionally Fermented African Locust Bean Condiment. *Global J Biotech & Biochem* 2008;3(1):42-46.

[15] Hwang KJ, Choi KH, Kim MJ, Park CS, Cha J. Purification and characterization of a new fibrinolytic enzyme of *Bacillus licheniformis* KJ-31, Isolated from Korean traditional *Jeotgal. J Microbiol Biotechnol* 2007;9:1469–1476.

[16] Mahmoud MG, Ghazy IA, Ibrahim GS, Fahmy AF, El-Badry MO, Abdel-Aty AM. Purification and characterization of a new fibrinolytic enzyme of *Bacillus polymaxa* NRC-A. *International journal of academic research* 2011;3(4): 542-547.

[17] Kim GM, Lee AR, Lee KW, Park JY, Lee MR, Chun J, Cha J, Song YS, Kim JH. Characterization of a 27kDa fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH51 isolated from *Cheonggukjang. J Microbiol Biotechnol* 2009;19(9): 997-1004.

[18] Agrebi R, Haddar A, Hajji M, Frikha F, Manni L, Jellouli K, Nasria M. Fibrinolytic enzymes from a newly isolated marine bacterium *Bacillus subtilis* A26: characterization and statistical media optimization. *Canadian Journal of Microbiology* 2009;55(9): 1049-1061.

[19] Wang C, Du M, Zheng D, Kong F, Zu G, Feng Y. Purification and Characterization of Nattokinase from *Bacillus subtilis* Natto B-12. *J Agric Food Chem* 2009;57:9722-9729.

[20] Ko JH, Yan JP, Zhu L, Qi YP. Identification of two novel fibrinolytic enzymes from *Bacillus subtilis* QK02. *Comp Biochem Physiol C Toxicol Pharmacol* 2004;137:65–74.

[21] Peng Y, Huang Q, Zhang R, Zhang Y. Purification and characterization of a fibrinolytic enzyme produced by *Bacillus emyloliquefaciens* DC-4 screened from *douchi*, a traditional Chinese soybean food. *Biochem Mol Biol* 2003;134:45-52.

[22] Paik HD, Lee SK, Heo S, Kim SY, Lee H, Kwon TJ. Purification and characterization of the fibrinolytic enzyme produced by *Bacillus subtilis* KCK-7 from *Chungkookjang*. *J Microbiol Biotechnol* 2004;14(4): 829–835.

Presented at ISFA (September 16-17, 2014-Semarang, Indonesia) as paper #118, ”Managing Biosafety and Biodiversity of Food from Local to Global Industries”